THE REGULATION OF MAMMARY CELL GROWTH

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Abstract

Changes in modern lifestyle such as lack of sleep, stress, and light exposure late at night are associated with increased rates of breast cancer. Most physiological processes, including growth, development, and metabolism, are controlled by circadian clocks. Circadian clocks respond to environmental cues to synchronize internal physiological processes, and thus, the disruption of this system may be responsible for this connection. The master clock in the brain coordinates peripheral circadian clocks located in every tissue of the body, including the mammary gland, and the core circadian component CLOCK regulates circadian oscillation of gene expression. Currently, the physiological function of the mammary clock, and the role of CLOCK in mammary cell growth and differentiation, are unknown. Our objective was to determine if the molecular clock controls mammary epithelial cell growth. shRNA specific for *Clock* was transfected into a normal mouse mammary epithelial cell line, HC-11. Q-PCR and western blot analysis showed sh*Clock* transfection significantly reduced *Clock* mRNA and protein abundance. Temporal analysis of molecular clock gene *Per1* showed loss of circadian oscillation in sh*Clock* transfected cells, indicating loss of molecular clock function. Cyclin D1 (*Ccnd1*) expression was elevated in sh*Clock* transfected cells, and growth curve analysis revealed that sh*Clock* transfected cells had significantly shorter doubling time than HC-11 control cells. These findings support that CLOCK regulates mammary epithelial cell growth and suggests that disruption of circadian clock mechanisms may lead to cancer by altering cell growth regulation.

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THE REGULATION OF MAMMARY CELL GROWTH:

Determining the Role of *Clock*

Emily Erickson, Biochemistry

INTRODUCTION

As the world continues to progress in medicine and health, cancer remains one of the diseases that society has yet to develop successful treatments and preventative measures against. In the United States, the most common form of cancer in women is breast cancer, which accounts for over 14% of all new cancer cases each year (National Cancer Institute, 2014). Interestingly, the incidence of breast cancer has dramatically increased in industrialized nations (Stevens, 2009). Changes in lifestyle due to industrialization, such as lack of sleep, stress, and light exposure late at night, may play a role in the increase in cancer incidence (Stevens, 2009). The human body possesses a circadian system to link external cues, for example, a light-dark cycle that results from the rotation of the Earth every 24 hours, with internal physiological processes. Changes in lifestyle, such as shift work and jetlag, which are inconsistent with external cues familiar to humans, disrupt the circadian system (Albrecht & Eichele, 2003). Shift work involving a circadian disruption was recently classified as "probably carcinogenic" by the International Agency for Research on Cancer (Bonde et al., 2012). A better understanding of the mechanisms governing molecular clocks and how they regulate physiology may lead to the discovery of links between circadian disruptions and cancer. The research reported herein investigates the role of a core circadian clock gene, *Clock*, in regulation of mammary epithelial cell growth.

Circadian rhythms are 24-hour (h) cycles in behavior and physiology (e.g., sleep-wake cycles, plasma hormone levels, and body temperature) that evolved to coordinate an organism's internal physiological processes with its external environment (Albrecht & Eichele, 2003). The master circadian clock is located in the suprachiasmatic nuclei (SCN) of the brain and functions to coordinate the timing of peripheral circadian clocks located in every tissue of the body (Bonde et al., 2012). One of the primary environmental cues the master clock responds to is light-dark cycles. The master clock translates photoperiod signals into neural and hormonal cues that serve as inputs to clocks in peripheral tissues (Dahl, Tao, & Thompson, 2012). Molecular clocks within each tissue generate circadian rhythms of gene expression, which then translate into physiological and metabolic rhythms.

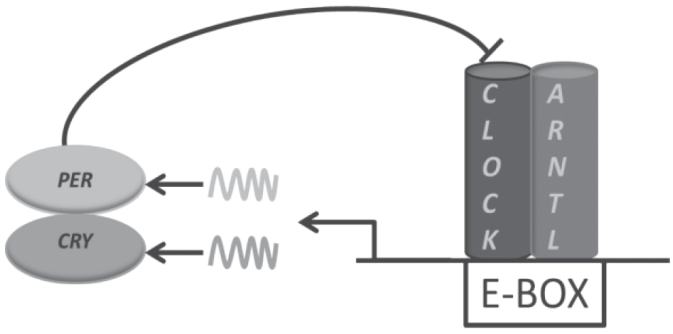

Figure 1. Mechanism of circadian rhythm generation by molecular clocks. See text for details.

At the molecular level, circadian rhythms are generated through a transcription-translation feedback loop composed of a set of core clock genes. Circadian locomotor output cycles kaput (*Clock*) and aryl hydrocarbon receptor nuclear translocator-like (*Arntl*) are among the core clock genes. As seen in Figure 1, CLOCK and ARNTL proteins come together to form a transcription factor that binds to an E-box sequence. E-box sequences are nucleotide sequences in the promoter region of a gene that transcription factors

bind to in order to regulate expression of the gene. The CLOCK:ARNTL transcription factor binds to the E-box sequence in the promoter region of the core clock genes *Period (Per1, Per2, Per3)* and *Cryptochrome (Cry1, Cry2)* and initiates their transcription. Together, PER and CRY proteins inhibit the formation of the CLOCK:ARNTL dimer, thus repressing their own transcription. Besides regulating *Per* and *Cry* genes, the CLOCK:ARNTL transcription factor also has the potential to bind E-box sequences in the promoter region of other genes (i.e., clock-controlled genes), resulting in circadian oscillation in 5–10% of genes expressed in every tissue of the body (Casey & Plaut, 2012).

Nearly all physiological systems in the body are regulated by circadian clocks, including the regulation of cell growth. Cell cycle progression occurs at specific times of the day and night, and in many organs, the Gap1/ Synthesis (G1/S) checkpoint of the cell cycle is expressed rhythmically, suggesting that the circadian system may play a role in its regulation (Fu, Patel, Bradley, Wagner, & Karsenty, 2005). In support of circadian clock regulation of cell growth is the finding that cyclin D1 (*Ccnd1*), which regulates progression of the cell cycle at the G1/S transition, is elevated in *Period1* and *Period2* knockout mice (*Per1-/-* and *Per2m/m,* respectively). Osteoblasts from *Per1-/-* and *Per2m/m* mice had shorter cell cycles and increased rate of cell growth (Fu et al., 2005). This supports the idea that the cell cycle is controlled in part by components of the circadian clock through regulation of *Ccnd1* and likely other factors (Matsuo et al., 2003).

A common method for determining the function of a gene is to reduce the expression of that gene. This can be done by transfecting short hairpin RNA (shRNA) into cells. Transfection is the process of introducing nucleic acids, such as shRNA, into a eukaryotic cell. The shRNA is a sequence of RNA that is complementary to the target gene of interest (Rao, Vorhies, & Senzer, 2009). Once transfected into the cell, shRNA integrates into the genome and begins to transcribe the shRNA product. The shRNA product is processed by cellular machinery (Dicer and the RNA-induced silencing complex [RISC]) to produce an activated RISC that contains a single strand of small interfering RNA (siRNA). siRNAs target and cleave complementary mRNA, which decreases the expression of the target gene (Rao et al., 2009). To study circadian clock regulation of mammary growth, shRNA specific for the mouse gene *Clock*, sh*Clock,* was transfected into a mouse mammary epithelial cell line, HC-11. The goals of this study were: (a) to reduce *Clock* expression in HC-11 cells by transfecting sh*Clock* into cultures; (b) to measure the effect of sh*Clock* on CLOCK protein abundance; and (c) to determine whether sh*Clock* impacts growth of cells using growth curve analysis.

EXPERIMENTAL PROCEDURES

HC-11 Cell Culture Maintenance and Passaging

HC-11 cells were cultured in complete growth medium (RPMI 1640 supplemented with 2g/L sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% heat inactivated calf serum, 5 µg/mL insulin, and 10 ng/mL epidermal growth factor [EGF]) in 5% CO_2 at 37°C. Cell culture media was changed every two days. Cells were rinsed once with sterile phosphate buffered saline (PBS) prior to media change. Cells were passaged, or split, when they reached 80% confluence (i.e., when cells filled 80% of the bottom of the culture dish). Cells were passaged 1:3 for maintenance by incubation in 0.25% Trypsin EDTA.

shRNA Transfection and Selection of Dilutional Clones

Four unique Qiagen SureSilencing shRNA plasmid sequences specific to the mouse *Clock* gene (Table 1) were transfected into HC-11 cells. Each shRNA plasmid carried a U1 promoter, an shRNA sequence, and a gene for hygromycin resistance.

Sequence ID	Insert Sequence
1	AAACCCACATTCCTTAGTAAT
2	GCAACTTGTGACCAAATTAGT
3	CGATGTCTCAAGCTGCAAATT
4	ATCAAACCCTGGATTGAATTT
Neg Ctrl	ggaatctcattcgatgcatac

Table 1. Identification of Plasmid Sequences.

Plasmids were introduced into *E. cloni* chemically competent bacterial cells, which were used to amplify the quantity of plasmid for future use in transfections. The plasmid was then isolated from the bacterial cells following Qiagen's instructions for the Endofree Plasmid Maxi kit. Next, the plasmids were linearized by restriction digest using the enzyme BsaI-HF and kit (New England BioLabs, Inc.).

A hygromycin dose response curve was used to determine the lethal dose of hygromycin for nontransfected cells (concentration needed to kill 100% of nonresistant cells). Cells were grown to confluence and then wells were treated in duplicate with varying concentrations of hygromycin (100, 200, 400, 600, 800, and 1,000 µg/mL). Media was changed every 48 hours, and wells were monitored for signs of cell death starting on day 7. The lethal dose of hygromycin was determined to be 200 µg/mL.

For transfection, HC-11 cells were plated at a density of 100,000 cells/mL in 24-well cell culture plates with

complete growth media. Linearized *Clock* shRNA plasmids were transfected into HC-11 cells using the lipofection transfection reagent Attractene (Qiagen cat. no. 1051561). Transfections were optimized by varying timing of exposure to transfection complexes following the manufacturer's protocol. Transfection complexes (0.3 µg linearized DNA, Optimem I Reduced Serum Media [Life Technologies] to a total of 25 µl, and 1.13 µl of the Attractene Transfection Reagent) were added to each well. Transfection was performed in duplicate for each plasmid sequence.

After transfected cells reached confluence, hygromycin was added at the lethal concentration for nontransfected cells (200 µg/mL). As a result, cells viable after 7 days of hygromycin selection contained the plasmid. To ensure continued selection of cells carrying plasmids, media was supplemented with maintenance strength of hygromycin (50 µg/mL).

The effect of plasmid transfection on the level of *Clock* mRNA expression was screened using RT-qPCR. Based on lowest levels of *Clock* mRNA expression, two lines of HC-11 cells were selected for further experimentation. These lines carried Sequence 2 and Sequence 4 plasmids. Nontransfected HC-11 cells served as wild-type control. To achieve a more consistent level of *Clock* mRNA expression, and to increase experimental repeatability, one cell from each plasmid sequence was isolated and grown into a clonal population using dilutional cloning, a process of serial dilutions used to obtain a cell culture well containing only one cell, which can then be grown into a population of clonal cells.

RNA Isolation and Real-Time Quantitative PCR Analysis (RT-qPCR)

Transfected cells and wild-type HC-11 cells were plated in duplicate wells at 100,000 cells/well in 35mm^2 dishes in complete growth media. Molecular circadian clocks were then synchronized by media change once cells reached confluence. RNA was isolated from cultures 4 hours and 16 hours after synchronization. Previous studies showed that these time points were equivalent to peak and trough levels of core clock gene expression (Casey et al., 2014). RNA was collected from cells and isolated using Qiagen's RNeasy kit, and it was evaluated for quality using the Nanochip (Agilent, Inc.'s Bioanalyzer 2100) and quantity using Nanodrop.

Gene expression was analyzed using TaqMan One-Step RT-qPCR (Life Technologies). Mouse-specific gene expression assays were used to measure the expression of *Clock* (cat. no. Mm00455950_m1), *Per1* (cat. no. Mm00501813_m1), *Ccnd1* (cat. no. Mm00432359_m1), and *β-actin* (cat. no. Mm00607939_s1) in lines carrying shRNA insert Sequences 2 and 4, relative to the expression in nontransfected HC-11 controls.

Knock-down (Kd), or reduction of *Clock* expression, was calculated using Equation 1.

Equation 1.

$$Clock_{KD} = 100 * (1 - 2^{\Delta\Delta CT})$$

where $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{negative\ control}$

$$\Delta CT_{sample} = CT_{sample} - CT_{reference}$$

Note: "Negative control" designates nontransfected HC-11 cultures.

In RT-qPCR, the cycle threshold (CT) is the cycle number in which the gene of interest exceeds the threshold value, and it is used to calculate relative gene expression (Applied Biosystems, 2008). Relative expression (RQ) of *Clock, Per1,* and *Ccnd1* were calculated with β-actin as the reference gene, using the delta-delta CT method. Expression was normalized to the average expression in wild-type HC-11 cells across 4-hour and 16-hour time points. Data are presented as log base 2 of relative expression levels.

Protein Isolation and Western Blot Analysis

Protein was harvested to determine the effect of sh*Clock* on abundance of CLOCK protein. Duplicate wells of each clonal line were plated at 700,000 cells per 100 cm^2 cell dish and grown to confluence. Protein lysates were isolated from cultures by pouring off media, washing cells two times with chilled PBS, and adding 500 µl of NP40 Protein Extraction Buffer (150 mM NaCl, 1.0% Nonidet-P40, 50 mM Tris-HCl pH 8.0, and protease inhibitors) to each dish. Cell lysates were then harvested using a chilled scraper and transferred to a 1.5 mL microfuge tube. To further aid cell lysis so that proteins could be isolated, the suspension was drawn up and expelled through a 26.5 gauge 1 ml syringe. Tubes were then shaken for 30 minutes on a VWR Mini Vortexer, followed by centrifugation for 20 minutes at 16,000 g at 4°C. Protein was then aliquotted into microfuge tubes and stored at -80°C until further use. Protein concentration was measured using the Quick Start Bradford Protein Assay (Biorad).

Fifteen µg of protein was loaded per lane and electrophoresed on a 10% TGX precast SDS PAGE gel from BioRad. The protein was then transferred onto a Polyvinylidene fluoride (PVDF) membrane for western blot analysis. Membranes were probed for CLOCK and B-ACTIN proteins using Anti-CLOCK (AbCam, ab3517) and Anti-B-ACTIN (AbCam ab8227) antibodies

at dilutions of 1/5,000 and 1/7,500, respectively. The secondary antibody (ab97051) was then incubated on the blots at the dilution of 1/100,000. Membranes were washed with the detection reagent ECL Plus substrate solution (GE Healthcare). Blots were imaged using the Typhoon imaging system, and the relative band intensity was measured.

Growth Curve Analysis

A growth curve analysis was used to determine if CLOCK affects HC-11 cell growth characteristics. Wild-type HC-11 cells, and Sequence 2 and Sequence 4 transfected cells were plated in duplicate wells at 100,000 cells/well in 35mm^2 dishes in normal growth media. Duplicate wells were counted on days 2, 4, 6, and 8, with media changed every 2 days. Cells were trypsinized, pelleted by centrifugation, and resuspended in sterile PBS. Trypan blue (0.4%, Sigma-Aldrich) was added in a 1:1 ratio to the cell suspension for live-dead staining. Each well was counted twice for live cells using a BioRad TC20 cell counter. Mean values of the technical and biological replicates and standard deviation of cell counts were graphed. The doubling times of cells during exponential growth were calculated using Equation 2:

Equation 2.

$$Doubling\ Time = \frac{T * ln2}{\frac{Xe}{Xb}}$$

Where T is the incubation time in any units, Xb is the cell number at the beginning of the incubation, and Xe is the cell number at the end of the incubation time. Growth curve experiments were repeated three times.

Data and Statistical Analysis

Data are presented as mean ± standard error (SE). A statistical analysis was performed using Minitab software. A student t-test was used to analyze whether shRNA sequences significantly impacted variables (relative expression, protein abundance, doubling time, and cell density) relative to wild-type HC-11 cultures. General linear model was used to determine the effect of treatment and time on cell number. A Tukey's test was used for post hoc pairwise comparisons. Significance was considered $P \leq 0.05$.

RESULTS

RT-qPCR analysis of HC-11 cells transfected with sh*Clock* Sequence 2 and Sequence 4 revealed a ~70% and ~30% reduction in *Clock* mRNA expression, respectively, relative to wild-type HC-11 control cells. Western blot analysis was used to determine if decrease in *Clock* mRNA levels affected CLOCK protein abundance. Analysis of western blot band intensity showed a 20% and 9% reduction in CLOCK to B-ACTIN ratio relative to wild-type HC-11 cultures (Figure 2). A student t-test comparing wild-type HC-11 and Sequence 2 and Sequence 4 transfected cell lines showed that CLOCK/B-ACTIN ratio was significantly less in Sequence 2 transfected cells, and thus support that CLOCK protein abundance was lower in cultures carrying Sequence 2 sh*Clock*. However, the CLOCK protein abundance was not significantly different ($P > 0.05$) between wild-type HC-11 cultures and Sequence 4 transfected cells.

RT-qPCR analysis was used to determine the effect of shRNA sequences on expression of *Clock, Per1,* and

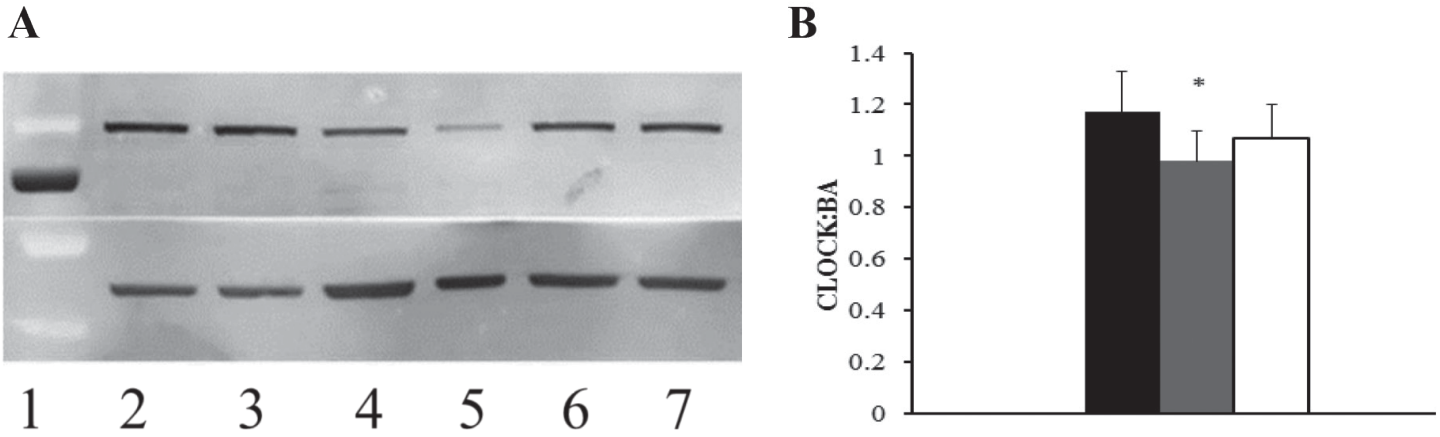

Figure 2. Western blot analysis of the impact of shRNA sequence on CLOCK protein abundance. Western blot analysis of CLOCK expression in wild-type HC-11 (lanes 2 and 3) cells, and HC-11 cells transfected with Sequence 2 (lanes 4 and 5) or 4 (lanes 6 and 7) sh*Clock*, and protein ladder in lane 1 (A). Total protein was collected after cells had been grown to confluence. Protein was electrophoresed on an SDS PAGE gel and then transferred to a PVFD membrane for western blotting. Membranes were probed using anti-CLOCK and anti-B-ACTIN, followed by incubation with secondary antibody, which was visualized by incubating with ECL-Plus. Blots were imaged using the Typhoon imaging system, and the relative band intensity was measured. CLOCK:B-ACTIN ratios are reported from wild-type HC-11 (black bar) and HC-11 transfected with Sequence 2 (gray bar) or Sequence 4 (white bar) (B). Values are mean ration ± SE; *indicates a difference at $P < 0.05$.

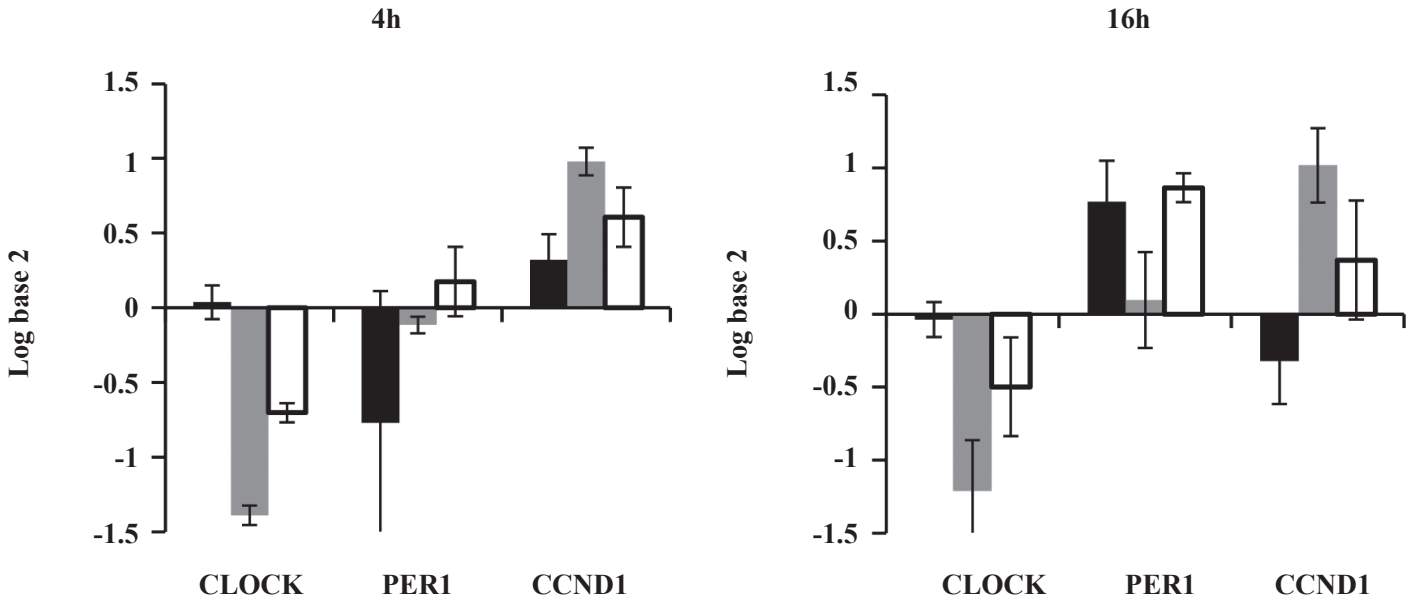

Figure 3. Impact of shRNA sequence on expression of *Clock, Per 1, and Ccnd1* in HC-11 cells at 4-hour and 16-hour circadian time points. Cells were grown to confluence, cultures were synchronized by media change, and RNA was isolated from cells 4 hours and 16 hours later. Expression of *Clock, Per1,* and *Ccnd1* were measured in HC-11 wild-type cultures (black) and cultures transfected with Sequence 2 (gray) and Sequence 4 (white) shRNA using RT-qPCR; values are mean ± SE.

Ccnd1 genes at 12-hour intervals after synchronization (Figure 3). At both 4 hours and 16 hours, *Clock* expression was decreased in cultures transfected with Sequence 2 shRNA relative to wild-type HC-11 cultures. *Per1* expression showed a large change in expression in HC-11 cells between 4-hour and 16-hour time points, oscillating in relative expression from 0.77 ± 0.20 to -0.77 ± 0.62. There was little to no change in *Per1* expression levels between 4 hours and 16 hours in Sequence 2 transfected cells, varying from -0.11 ± 0.23 to 0.11 ± 0.04. In lines carrying Sequence 4, sh*Clock* relative expression of *Per1* varied from 0.34 ± 0.070 to -0.34 ± 0.16.

Ccnd1 expression in control HC-11 cells varied from -0.32 ± 0.21 at 4 hours and 0.32 ± 0.12 at 16 hours. HC-11 cells transfected with Sequence 2 levels of *Ccnd1* did not change between 4-hour and 16-hour time points (0.019 ± 0.18 and -0.019 ± 0.065). Levels of *Ccnd1* changed from a relative expression of -0.12 ± 0.29 at 4 hours to 0.12 ± 0.14 at 16 hours in HC-11 lines carrying Sequence 4 shRNA. Interestingly, levels of *Ccnd1* were elevated in lines carrying shRNA Sequence 2 relative to wild-type lines at both time points

Growth curves revealed that cultures transfected with shRNA Sequence 2 had reached almost a twofold higher cell density (3,423,158 cells/35mm^2 ± 160,862 cells) compared to control HC-11 cells (1,867,020 cells/35mm^2 ± 1,174,727 cells) within 8 days (Figure 4). Cells grew similarly in lag phase until day 4, when they entered log phase of growth. Log phase doubling time between days 4 and 6 was 43 hours for Sequence 2, and doubling

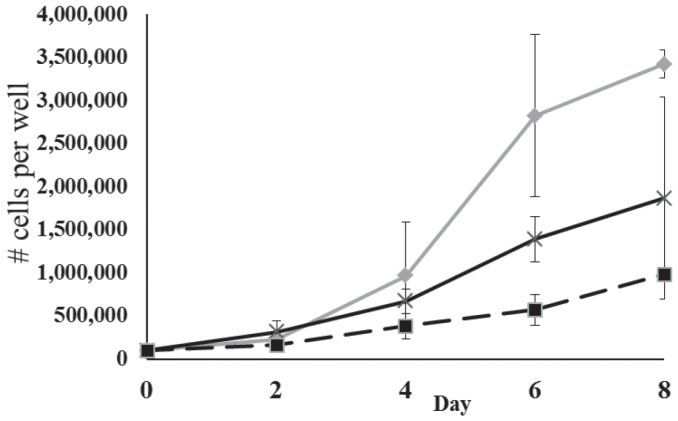

Figure 4. Analysis of the impact of sh*Clock* on HC-11 growth curves. Wild-type HC-11 (black), Sequence 2 (gray), and Sequence 4 (dashed) cells were plated at 100,000 cells/35mm dish. Duplicate wells were harvested from each line on days 2, 4, 6, and 8, and cells were stained with Trypan blue and counted for live cells using a BioRad TC20 cell counter. Mean values of the technical and biological replicates and standard deviation of cell counts were graphed. The experiment was repeated three times. Treatment and day had a significant impact on growth ($P < 0.05$). Post hoc analysis found Sequence 2 growth significantly different ($P < 0.05$) from wild-type and Sequence 4, but no difference between wild-type and Sequence 4 growth ($P > 0.05$).

time for HC-11 cells was 64 hours over the same period. Doubling times were significantly different, with $P = 0.040$.

DISCUSSION

Clock mRNA expression was decreased in Sequence 2 shRNA transfected cells by ~70%, while in cultures carrying Sequence 4 shRNA *Clock* expression was reduced by 30%. Western blot analysis revealed CLOCK protein levels were significantly lower in the line carrying Sequence 2 as compared to nontransfected HC-11 cells (Figure 2). Although *Clock* gene expression was decreased in cells transfected with Sequence 4 sh*Clock,* levels of CLOCK protein were not different from HC-11 controls. Transcription and translation do not have a simple linear relationship (Maier, Guell, & Serrano, 2009). A difference in mRNA levels, but a lack of difference in protein levels, may be due to factors including mRNA stability, mRNA secondary structure, regulatory proteins, and protein half-lives (Maier et al., 2009). Thus, because lower levels of mRNA do not necessarily confirm a low amount of CLOCK protein, it was necessary to show that CLOCK protein levels were decreased in lines transfected with sh*Clock*. These data show that the level of CLOCK protein were decreased in Sequence 2 transfected cells relative to HC-11 cells, but Sequence 4 did not significantly impact CLOCK protein abundance.

Differences in effectiveness of Sequence 2 and Sequence 4 shRNA in decreasing levels of CLOCK mRNA expression and protein abundance may be the result of nucleotide sequence variation of shRNA inserts (Rao et al., 2009). Thus, it is likely that the Sequence 2 plasmid sequence more effectively targeted the *Clock* mRNA transcript and therefore more effectively prevented it from being translated into protein. Alternatively, off-target effects that may have led to variations in expression reduction may have been due to differences in the location and number of integrations of shRNA sequence into the genome, or due to an immunological response to the introduction of dsRNA (Rao et al., 2009). Together, these data support that the nucleotide of Sequence 2 sh*Clock* significantly reduced CLOCK protein abundance, while Sequence 4 had minimal to no effects on CLOCK protein levels.

In addition to analyzing the gene expression of *Clock,* we also measured the expression of *Per1* and *Ccnd1*. Previous studies in our lab showed that HC-11 cells have a functional clock, as the core clock genes *Arntl, Per1, Per2*, and *Cry1* exhibit circadian rhythms of expression, with peak and trough of expression of *Per1* occurring at 4 hours and 16 hours after media change (Casey et al., 2014). Consistent with this finding, *Per1* levels exhibited a 3.4-fold difference in expression levels between 4-hour and 16-hour time points in wild-type HC-11 cultures, supporting a functional molecular clock. In contrast, *Per1* did not oscillate between 4-hour and 16-hour time points in HC-11 cultures carrying Sequence 2 shRNA levels, supporting that significantly decreasing CLOCK protein abundance effectively eliminated the function of the molecular clock. In cultures carrying Sequence 4, shRNA levels of *Per1* varied by only 0.69-fold, suggesting that this sequence may have somewhat diminished the function of the molecular clock.

Levels of *Ccnd1* expression oscillated between 4 hours and 16 hours in wild-type HC-11 cells. The molecular clock indirectly controls *Ccnd1* expression, so rhythmicity of *Ccnd1* was expected in wild-type cells (Fu et al., 2005). However, *Ccnd1* exhibited no significant change in expression between 4-hour and 16-hour time points in Sequence 2 transfected cells, and it was consistently higher in Sequence 2 transfected cells at both time points relative to wild-type HC-11 cultures.

Ccnd1 encodes a cell cycle molecule, cyclin D1, which regulates progression through the G1/S checkpoint of the cell cycle. Musgrove, Lee, Buckley, and Sutherland (1994) showed that increasing levels of cyclin D1 in breast cancer cells increased the rate of cell-cycle progression. Further, Wood and colleagues (2008) showed that down regulation of *Per1 and Per2* in mouse breast cancer cells increased *Ccnd1* expression and enhanced cell growth. We have demonstrated in this study that decreasing CLOCK abundance, which resulted in loss of *Per1* oscillation and consistently high levels of *Ccnd1,* increased the rate of cell growth in a normal mouse mammary epithelial cell line. In particular, Sequence 2 transfected cells had a faster doubling time than HC-11 cells. Together, these findings suggest that loss of molecular clock function increases *Ccnd1* expression levels across the day and leads to enhanced cell growth. These data support that the mammary clock regulates growth and development of the gland, and they demonstrate that loss of the circadian clock may lead to unregulated growth. Cancer is characterized by increased and uncontrolled cell proliferation, so a better understanding of the mammary circadian clock's role in this process has the potential for development of new preventative measures or treatments for breast cancer.

In future studies, we will further examine the connection between the gene *Clock* and the cell cycle. Future experiments will include more control cell lines, such as a working nontargeting scramble sequence, which is needed to show that any effects seen in gene or protein expression are not a result of the transfection procedure. Additionally, we hope to examine the

expression of other cell cycle regulatory genes believed to be controlled by the molecular clock, such as *Wee1* and *C-myc,* to determine if these genes are affected by shRNA targeting *Clock.*

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